Purpose

An introduction to the physiology of enzymes in biology.

Equipment

- 2 blue pipet aid
- 1ml Pipet for enzyme
- 1ml pipet for H_2O_2
- 5mL pipet
- cuvettes
- Kimwipe

Chemicals

- pH5 Buffer
- Guaicol
- H_2O_2
- Ice
- Peroxidase enzyme

Background

Enzymes are catalysts. They speed up chemical reactions without being used up in the process. They react with substrates to form a temporary intermediary complex from which the new product is released **??**. After the reaction, the unchanged enzyme is released and can again recombine with more substrate until the substrate is depleted. The enzymes of living cells greatly accelerate chemical reactions and by governing relative reaction rates, regulate overall directions of metabolic change.

$$\text{Enzyme} + \text{Substrate} \longrightarrow \text{Enzyme–Substrate Complex} \longrightarrow \text{Enzyme} + \text{Products}\ (\text{E})\ (\text{S})\ (\text{ES})\ (\text{E})\ (\text{P})$$

(Reaction En2.1)

Every enzyme acts as a specific catalyst, that is, it reacts with a specific substrate to catalyze a narrow class of chemical reactions. Living cells contain thousands of enzymes, each catalyzing unique reactions. In this exercise, we will study and report on the physiology of the enzyme: peroxidase.

This lab uses peroxidase extracted from vegetable tissue (usually turnip or horseradish). The normal function of peroxidase is to convert toxic hydrogen peroxide (H_2O_2) to water and oxygen. Peroxidase splits off oxygen, which then accepts hydrogen from another substrate molecule. During the same reaction, another molecule, picks up the H_2 released from H_2O_2 and is reduced. This is only one example of the many oxidation - reduction reactions important at the cellular level.

We can observe and measure the reaction by monitoring oxygen formation. We will use and oxygen reactive dye to visualize a colored end product. The dye guaiacol turns brown when oxidized. We will measure the reactivity

by a recording the change in absorbance at 500nm **??**. Higher enzyme activities make the solution turn brown faster. In this lab, the substrate added for the peroxidase extract to work on is hydrogen peroxide.

$$H_2O_2 + \text{Guaiacol} - H_2 \longrightarrow 2\,H_{20} + \text{Guaiacol (colorless) (colored)} \qquad \text{(Reaction En2.2)}$$

In this experiment, you will use various conditions of substrate concentration, and pH to study their effect on the enzyme's function.

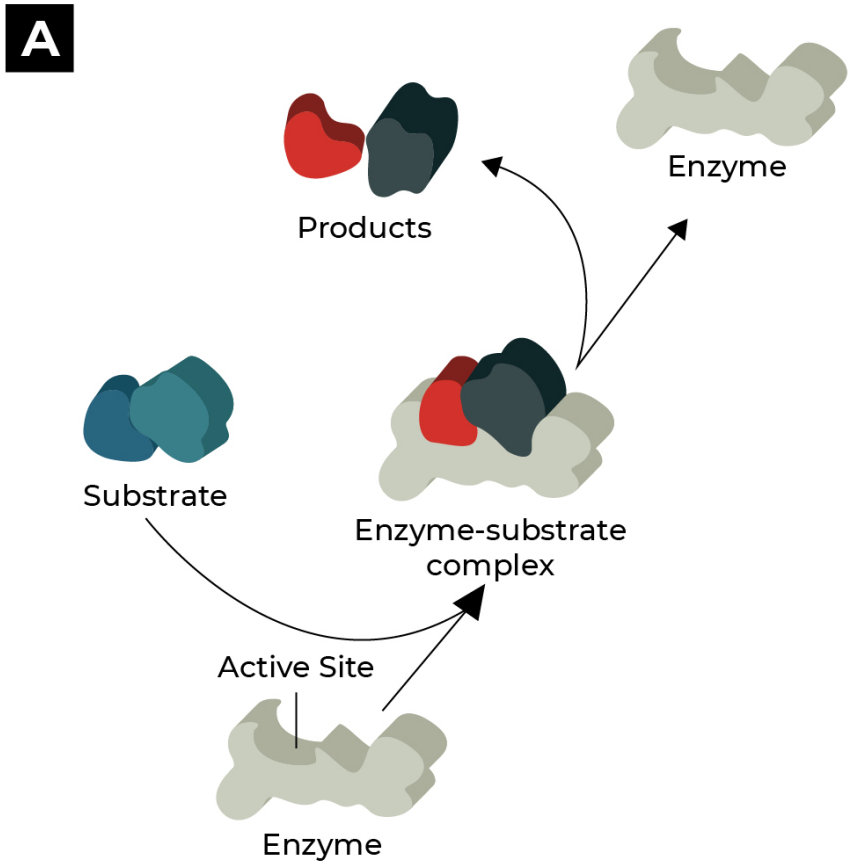

Figure En2.1: Biological Reaction Utilizing an Enzymes Catalytic Activity

Procedure

- 1 labeled blue pipet aid and 1mL pipet for enzyme extract
- 1 labeled blue pipet aid and 1mL pipet for H_2O_2
- use labeled 5mL pipet for pH5 buffer- DO NOT TAKE EXTRA BUFFER TO YOUR BENCH
- leave Guaiacol bottle in the hood and capped when not in use
- always add the enzyme last just before assaying each tube
- each tube must be assayed individually because of the close timing of the readings
- keep the extract on ice and all other reagents at room temperature
- number cuvettes with china marker near the top
- always wipe tubes with a Kimwipe before inserting
- rinse cuvettes with distilled water and tap dry on tissue after each use
- do not use a test tube brush

Enzyme Extract Preparation

1. Weigh 1g of peeled turnip or other vegetable and cut into small pieces.
2. Homogenize the tissue by grinding in a cold mortar with a small amount of sand.

   > A small amount of sand is the amount you can hold between your index finger and thumb.

   Add small amounts of up to 50ml of cold 0.1M phosphate buffer (pH7).
3. Filter the mixture through 2 layers of cheesecloth into a beaker on ice.
4. Wash with the remainder of the 50ml of buffer.
5. Combine all of the extracts into one class extract.

   > Your TA will then redistribute the combined extract. This will make data comparisons much simpler.
6. Dispose of the cheesecloth in the regular trash. Rinse out beaker and funnel with distilled water.
7. Make sure the enzyme extract the TA gives you is put on ice during the lab.

> The spectrophotometers are hooked up using an A/B switch box even if each pair has their own spectrophotometer. Make sure the dial is set to your computer if you are having trouble collecting data.

Spectrophotometer and Computer Preparation

1. Turn on Spec at least 15 minutes before collecting data.
2. Log on to UTAD
3. Start SpectroPro after the spectrophotometer has warmed up. The spectrophotometer must be completely warmed up and the switch box set to your computer.
    a. Press the scan button
    b. Press OK to COM1
    c. You will see a welcome screen with an example graph and data table.
4. Start EXCEL and save a file for your experiment. Name your file Lab 22_YourName.
5. Your experiment lasts 2 minutes and acquires 6 points per minute.
6. This is an absorbance vs. time experiment. Click the button on the tool bar that has "A vs. time".

Enzyme Standardization

The extract contains hundreds of different types of enzymes, including peroxidase. Only peroxidase, however, will react with H_2O_2. The activities of enzymes vary with size and age of the tissue, extent of homogenization and age of the extract. For example, an extract might have a large amount of peroxidase or only a little. There is no way to predict ahead of time how much vegetable is needed to get a usable amount of enzyme in your extract. A trial run will be performed to determine the correct amount of extract to use in further experiments.

Table En2.1: Mixing Table for Enzyme Standardization Assays

Temperature (C)	Tube Number	pH Buffer(ml)	H_2O_2(ml)	Guiaicol(ml)	Enzyme(ml)
Blank	1	2.5	1	0.5	0
Low	2	2.25	1	0.5	0.25
Medium	3	2	1	0.5	0.5
High	4	1.5	1	0.5	1

1. Fill 4 cuvettes as directed in Table En2.1.

    DO NOT ADD THE ENZYME YET!

2. Zero the spec at 500nm using Tube 1.

a. Put tube 1 in the spec and press the 0 ABS 100% T button on the spec.
b. When the spec has finished, press the done button on the SpectroPro screen.

3. Set Up Tubes 1-4 as directed.
4. When you are ready to read tube 2
   a. One partner will add the enzyme and invert the tube to mix using a piece of parafilm.
   b. The other partner will press the collect button in SpectroPro to begin collecting data.

      Make sure you have parafilm ready for your experiment.

   c. These 2 steps must be done quickly and in unison. As soon as the enzyme is added, invert to mix, put in spec and press collect.
5. Allow the experiment to run. After 2 minutes, enter the data in REPORT Table En2.1.
6. Get ready to run tube 3.

   Remember to add the enzyme only when you are ready to push the collect button. This time, SpectroPro will ask if you want to keep the last set of data. Don't. When you press this button, data collection will begin.

Graphing the results of enzyme standardization in EXCEL

SpectroPro does not allow for multiple experiments on one graph or the calculations required.

1. In EXCEL, label a column for minutes, low, medium, and high.
2. Highlight the columns, and click the chart wizard.
3. Choose XY scatter not a line graph.
4. Insert your new chart as an object on the same page as your graphs.
5. You should have all data for the tubes for an experiment on one graph.
6. Which amount of enzyme gave the most linear absorbance change from 0 to 1 in approximately 120 seconds? Use this amount of enzyme in all subsequent experiments.

Substrate concentration

Peroxidase works on the substrate hydrogen peroxide. Many enzymes show a pattern where low substrate concentrations slow the reaction rate down. The enzyme reaction rate increases as substrate concentration rate increases. However, above a certain concentration the enzyme reaction rate does not increase because the enzyme cannot process any more substrate per unit of time.

1. Label and fill cuvettes as shown in Table En2.2.

Table En2.2: Mixing Table for Substrate Concentration Assays

Concentration	Tube number	Buffer(ml)	$H_2O_2(ml)$	Guiaicol(ml)	Enzyme(ml)
0% H_2O_2	10	_____	1	0.5	_____
3% H_2O_2	11	_____	1	0.5	_____
6% H_2O_2	12	_____	1	0.5	_____
12% H_2O_2	13	_____	1	0.5	_____

Adjust the amount of buffer according to the amount of enzyme added. Total tube volume should be 4ml. Remember to add enzyme last and only when you are ready to take measurements.

2. When you are ready to read tube 10
    a. One partner will add the enzyme and invert the tube to mix using a piece of parafilm.

        Make sure you have parafilm ready for your experiment.

    b. The other partner will press the collect button in SpectroPro to begin collecting data.
    c. These 2 steps must be done quickly and in unison. As soon as the enzyme is added, invert to mix, put in spec and press collect.
3. Allow the experiment to run. After 2 minutes, enter the data in Table En2.2.
4. Get ready to run tube 11. Remember to add the enzyme only when you are ready to push the collect button. This time, SpectroPro will ask if you want to keep the last set of data. **Don't**. When you press this button, data collection will begin.

Graphing the results of concentration experiments in EXCEL

1. Label a column for minutes, 0% H_2O_2, 3% H_2O_2, 6% H_2O_2, and 12% H_2O_2.
2. Highlight the columns, and click the chart wizard.
3. Choose XY scatter not a line graph.
4. You should have all data for the tubes for an experiment on one graph.
5. Determine which data is linear for each tube.
6. Calculate the enzyme activity for the linear portion.

Subtract the initial absorbance reading from the final absorbance reading in the linear portion of the reaction. Divide by the number of seconds. This number is the enzyme activity in change in absorbance units per second

7. Make a bar chart with enzyme activities on the y-axis and concentration on the x-axis.

pH

Many of the bonds that hold the enzyme together are affected by the pH of the surrounding solution. Denaturation is the change in shape as the pH is altered. In a narrow pH range, the bonds are in the correct position for optimal enzyme function. Outside of this range the bonds shift and the enzyme loses its shape and has reduced function.

Table En2.3: Mixing Table for pH Assays

Concentration	Tube Number	Buffer(ml)	H_2O_2 (ml)	Guiaicol(ml)	Enzyme(ml)
pH 3	14	_____	1	0.5	_____
pH 5	15	_____	1	0.5	_____
pH 7	16	_____	1	0.5	_____
pH 9	17	_____	1	0.5	_____

1. Label and fill cuvettes as shown in Table En2.3.

Adjust the amount of buffer according to the amount of enzyme added. Total tube volume should be 4ml.

Remember to add enzyme last and only when you are ready to take measurements.

2. When you are ready to read tube 14
    a. One partner will add the enzyme and invert the tube to mix using a piece of parafilm.

    Make sure you have parafilm ready for your experiment.

    b. The other partner will press the collect button in SpectroPro to begin collecting data.
    c. These 2 steps must be done quickly and in unison. As soon as the enzyme is added, invert to mix, put in spec and press collect.
3. Allow the experiment to run. After 2 minutes, enter the data in Table En2.3.

4. Get ready to run tube 15. Remember to add the enzyme only when you are ready to push the collect button. This time, SpectroPro will ask if you want to keep the last set of data. **Don't**. When you press this button, data collection will begin.

Graphing the results of pH experiments in EXCEL

1. Label a column for seconds, pH3, pH5, pH7, and pH9.
2. Highlight the columns, and click the chart wizard.
3. Choose XY scatter not a line graph.
4. You should have all data for the tubes for an experiment on one graph.
5. Determine which data is linear for each tube.
6. Calculate the enzyme activity for the linear portion.

   Subtract the initial absorbance reading from the final absorbance reading in the linear portion of the reaction. Divide by the number of seconds. This number is the enzyme activity in change in absorbance units per second
7. Make a bar chart with enzyme activities on the y-axis and pH on the x-axis.

Tables

Report Table En2.1: Results of Enzyme Standardization Assays

Time (sec)	Time (min)	Tube 2	Tube 3	Tube 4
0	0			
10	0.167			
20	0.333			
30	0.5			
40	0.667			
50	0.833			
60	1			
70	1.17			
80	1.333			
90	1.5			
100	1.67			
110	1.83			
120	2			

Report Table En2.2: Results of Concentration Assays

Time (sec)	Time (min)	Tube 2	Tube 3	Tube 4
0	0			
10	0.167			
20	0.333			
30	0.5			
40	0.667			
50	0.833			
60	1			
70	1.17			
80	1.333			
90	1.5			
100	1.67			
110	1.83			
120	2			

Report Table En2.3: Results of pH Assays

Time (sec)	Time (min)	Tube 2	Tube 3	Tube 4
0	0			
10	0.167			
20	0.333			
30	0.5			
40	0.667			
50	0.833			
60	1			
70	1.17			
80	1.333			
90	1.5			
100	1.67			
110	1.83			
120	2			

Questions

1. Why do we have to repeat the enzyme standardization assays again? Why not just use values from last session?

2. What is the optimal substrate concentration for this enzyme?

a) Which substrate amount gave the highest enzyme activity? Why?

b) Is there an increase in enzyme activity for every increase in substrate amount? Why?

3. What is the optimal pH for this enzyme?

a) Which pH caused the highest enzyme activity? Why?

b) Which pH gave the lowest enzyme activity? Why?

Graphs in Excel. Include your data tables and graphs. Send them via e-mail to your TAs biosciences2.utoledo.edu account. Remember to use your official UT e-mail account. Name your file Lab 23_yourname. Label axes, include units, make a key or legend, and indicate linear portion for F. Enyme Standardization - graph with 3 enzyme concentrations plotted with x= time and y= absorbance Concentration Assays - graph with 4 concentrations plotted with x= time and y= absorbance pH Assays - graph with 4 pHs plotted with x= time and y= absorbance